Arch Microbiol (1987) 149:120–124

Archives of
Microbiology

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Voltage-dependent depolarization of bacterial membranes and artificial lipid bilayers by the peptide antibiotic nisin

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Abstract. The peptide antibiotic nisin is shown to disrupt valinomycin-induced potassium diffusion potentials imposed on intact cells of *Staphylococcus cohnii* 22. Membrane depolarization occurred rapidly at high diffusion potentials while at low potentials nisin-induced depolarization was slower suggesting that nisin requires a membrane potential for activity. This assumption was proven in experiments with planar lipid bilayers (black lipid membranes). Macroscopic conductivity measurements indicated a voltage-dependent action of nisin. The potential must have a trans-negative orientation with respect to the addition of nisin (added to the cis-side) and a sufficient magnitude (ca. −100 mV). With intact cells the threshold potential was lower (−50 to −80 mV at pH 7.5 and below −50 mV at pH 5.5). Single channel recordings resolved transient multistate pores, strongly resembling those introduced by melittin into artificial bilayers. The pores had diameters in the range of 0.2–1 nm, and lifetimes of few to several hundred milliseconds. The results indicate that nisin has to be regarded as a membrane-depolarizing agent which acts in a voltage-dependent fashion.

Key words: Nisin, mode of action – Peptide antibiotics – Membrane depolarization – Planar lipid bilayers ("black lipid membranes") – Multi-state pores

The peptide antibiotic nisin is produced by various strains of *Streptococcus lactis* (Lancefield Group N). It has found considerable application to prevent outgrowth of bacterial spores in canned food and to antagonize competing Gram-positive bacteria in dairy processes (Hurst 1981). The mechanism of the bacterial action of nisin is still controversial. A detergent-like effect (Ramseier 1960) as well as inhibition of murein synthesis (Reisinger et al. 1980) were discussed as the primary cause of cell death. However, structural similarities of nisin to the staphylococcin-like peptide Pep 5 (Sahl et al. 1985) as to their size, cationic nature, and content of the rare amino acid lanthionine, suggested a similar mode of action for both. We have shown recently that nisin (Ruhr and Sahl 1985) like Pep 5 (Sahl and Brandis 1983; Sahl 1985a) promotes a rapid efflux of small cytoplasmic compounds such as K^+ and amino acids from Gram-positive bacteria. As a consequence the membrane potential dropped dramatically. Thus, nisin may dissipate the membrane potential and ionic gradients across the cytoplasmic membrane by affecting its integrity. Furthermore, experiments with intact cells and cytoplasmic membrane vesicles indicated that nisin requires an energized membrane to exert its effect. However, this could not be verified with artificial asolectin liposomes (Ruhr and Sahl 1985).

To substantiate our view of nisin as a membrane depolarizing agent we investigated its influence on intact bacterial cells energized artificially with a valinomycin-induced K^+ diffusion potential. Further, we report on the ability of nisin to increase membrane conductance in a voltage-dependent fashion and to introduce transient multi-state pores into black lipid membranes.

Material and methods

Experiments with intact cells

The influence of nisin on the membrane potential of intact cells was tested with *Staphylococcus cohnii* 22. The strain, the culture conditions, and the estimation of membrane potentials by means of lipophilic cations were described in detail recently (Ruhr and Sahl 1985). Briefly, *S. cohnii* was grown in tryptone soya broth (Oxoid), washed, resuspended in buffer (N-morpholino propanesulfonic acid (Mops), 10 mM, pH 7.5 or sodium citrate, 10 mM, pH 5.5) and incubated at 30°C. To monitor the membrane potential 0.2 μCi of [^{14}C] tetraphenylphosphonium (TPP^+) was added. The proton gradient of intact cells was dissipated with 50 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Artificial potassium diffusion potentials were created by addition of 50 μM valinomycin to CCCP-treated cells. To obtain diffusion potentials of different magnitude varying concentrations of KCl were added to the incubation buffer.

Planar membrane experiments

Black lipid bilayer membranes (BLM) were formed as described in detail previously (Benz et al. 1978). Briefly, the instrumentation consisted of a Teflon chamber with two aqueous compartments connected by a circular hole. The holes had surface areas of either 1 mm^2 (for macroscopic conductance measurements) or 0.1 mm^2 (for single channel experiments). Membranes were formed across the holes by painting on a 1% solution of different lipids in *n*-decane or *n*-chlorodecane. For membrane formation diphytanoyl

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Abbreviations: BLM, Black lipid membranes; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DOPC, dioleoyl phosphatidylcholine; PS, phosphatidylserine; TPP^+, tetraphenylphosphonium cation

phosphatidylcholine (DiPhPC), dioleoyl phosphatidylcholine (DOPC), phosphatidylserine (PS) (Avanti Biochemicals, Birmingham, AL, USA) were used. The aqueous 1 M KCl solutions (Merck, Darmstadt, FRG) were unbuffered or were buffered with 10 mM Mops (pH 7). The temperature was kept at 25° C throughout.

The membrane current was measured with a pair of calomel electrodes switched in series with a voltage source and an electrometer (Keithley 602). In the case of the single channel recordings the electrometer was replaced by a Keithley 427 current amplifier. The amplified signal was monitored with a storage oscilloscope (Tektronix 5115) and recorded with a tape or strip chart recorder. The orientation of the voltage was defined with respect to the addition of nisin (the cis-side). A trans-negative potential (indicated by a minus sign) means that a negative potential was applied to the compartment opposite to the addition of nisin.

Chemicals

Basic chemicals were purchased from Merck (Darmstadt, FRG), radiochemicals from Amersham-Buchler (Braunschweig, FRG), and biochemicals from Sigma (München, FRG). The phospholipids used for preparations of black lipid membranes were from Avanti Biochemicals (Birmingham, AL, USA). Nisin was obtained from Koch & Light (Colnbrook, England) and used after purification on reversed phase high performance liquid chromatography as described for the staphylococcal peptide Pep 5 (Sahl et al. 1985).

Results

Influence of nisin on artificially energized bacterial cells

Recent experiments strongly implicated the cytoplasmic membrane of Gram-positive bacteria as the primary target of nisin and indicated a requirement for an energized membrane for nisin action (Ruhr and Sahl 1985). However, it was not possible to define more precisely what the actual requirement was. The channel-forming colicins (for reviews see Konisky 1982; Cramer et al. 1983; Davidson et al. 1984) and the bee venom toxin melittin (Kempf et al. 1982; Tosteson and Tosteson 1984) were shown to need a transmembrane potential for membrane disruption. Assuming a similar requirement for nisin, an artificial valinomycin-induced potassium diffusion potential should be sufficient to promote nisin action. Therefore, we designed the following experiment: *Staphylococcus cohnii* 22 was incubated in K^+-free buffers and treated with CCCP to dissipate the proton gradient. Then the potassium carrier valinomycin was added to the cell suspension. The intracellular K^+ (concentration ca. 230 mM) leaked out because of the high permeability of the membrane in the presence of valinomycin while counterions were retained inside the cells. This resulted in an asymmetric charge distribution on both sides of the membrane and in a diffusion potential with a negative sign inside the cells. The actual magnitude of the diffusion potential could be manipulated by addition of defined K^+ concentrations to the outside. After energization with valinomycin the cells were treated with nisin. In the course of such an experiment samples were collected to determine $\Delta\Psi$.

Figure 1A demonstrates that valinomycin-induced diffusion potentials depending on the outside K^+ concentrations could be generated and that nisin was able to dissipate such diffusion potentials presumably by making the membrane permeable to counterions. The velocity of dissipation as indicated by the slope of the curve after nisin addition was depending on the magnitude of the preexisting potential. Figure 1 also shows that nisin was more active at acidic pH. While the level of $\Delta\Psi$ after treatment with nisin remained at ca. -50 mV when the cells were incubated at pH 7.5, it decreased further at pH 5.5 slowly approaching zero level. A similar observation was made when cells were treated with nisin without valinomycin addition (data shown in the left panel of Fig. 1A, B). After CCCP treatment $\Delta\Psi$ leveled off at ca. -50 mV to -60 mV (Fig. 1). Such treated cells could not be further influenced by nisin at pH 7.5, while at pH 5.5 $\Delta\Psi$ dropped to zero.

The influence of pH on the activity of nisin is in contrast to the situation observed with the staphylococcin-like peptide Pep 5 (Kordel et al., unpublished work). The activity of Pep 5 is strongly inhibited at acidic pH. Furthermore, it was possible to demonstrate a threshold potential for Pep 5 action of approximately -80 to -100 mV at pH 7.5 using similar experiments as described above. For nisin such a defined threshold potential cannot be derived from Fig 1. However, it may be in the range of -50 to -80 mV at pH 7.5 and below -50 mV at pH 5.5.

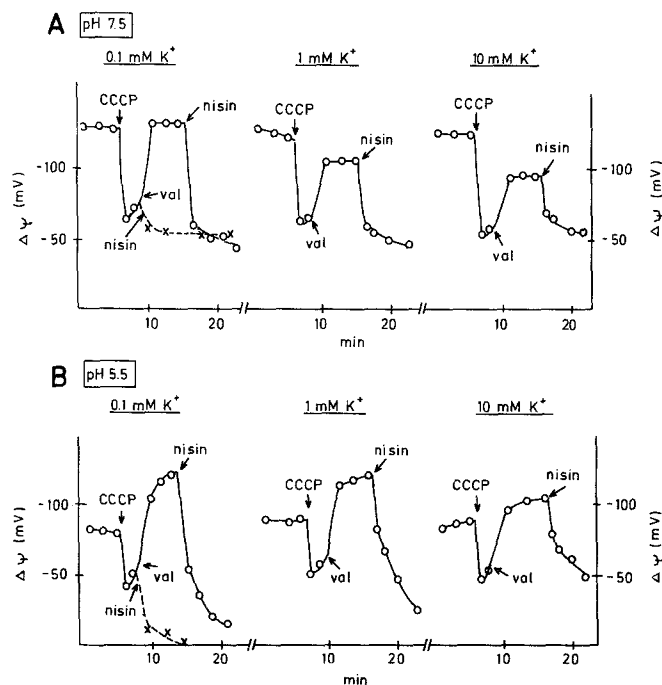

Fig. 1 A, B. Influence of the peptide antibiotic nisin on the membrane potential ($\Delta\Psi$) of *Staphylococcus cohnii* 22 artificially energized with valinomycin-induced potassium diffusion potentials. Cells were suspended in buffer at pH 7.5 (**A**) and 5.5 (**B**), uncoupled with CCCP, energized with valinomycin and finally treated with nisin. The magnitude of $\Delta\Psi$ was controlled by addition of KCl to the incubation buffer. Throughout the experiments $\Delta\Psi$ was measured by following the distribution of the lipophilic cation TPP^+ (O——O); the method is detailed by Ruhr and Sahl (1985). In the left panels of **A** and **B** the influence of nisin on cells not energized with valinomycin is shown (×– – – –×). For this purpose the assay was divided, one part receiving valinomycin and the other receiving nisin. The concentrations were 50 μM CCCP, 20 μM valinomycin and 3 μM nisin

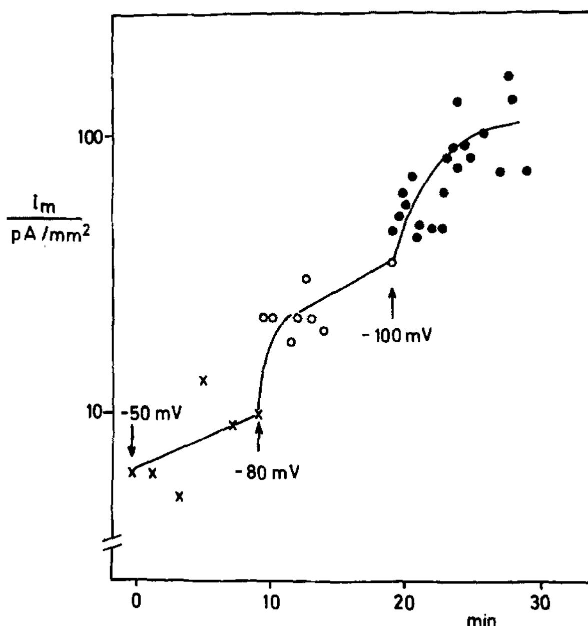

Fig. 2. Time course of trans-membrane currents in the presence of nisin (0.3 μM, cis-side only) at different voltages. The membrane was formed from DOPC/PS (4:1) dissolved in *n*-decane (1%, w/v); the membrane area was 1 mm^2

Planar membrane experiments

The results described above are consistent with the interpretation that the only prerequisite for nisin action is the existence of an electrical field of sufficient strength within the membrane. Therefore, we studied the influence of the peptide on black lipid membranes (BLM).

Macroscopic conductance measurements

In a first approach we measured the macroscopic membrane conductance in relation to the orientation of the applied voltage. When the potential was positive at the trans-side of the membrane (the opposite side of the addition of nisin) no increase of the specific membrane conductance was observed after addition of nisin. For trans-negative potentials up to −50 mV only a small increase of the membrane conductance as compared with the conductance in the absence of nisin was observed (Fig. 2). However, increasing the voltage to −80 mV and further to −100 mV led to considerably higher membrane currents. Figure 3 represents a current-voltage-plot which demonstrates the voltage-dependence of nisin action for increasing and decreasing voltages.

Figure 3 clearly shows that the membrane conductance is increasing with voltage but not in a linear fashion. The rise is slow and almost linear up to −100 mV but much faster above −100 mV. This again could point to a threshold potential for significant action in the range of −100 mV with artificial membranes. Decreasing the voltage led to reduced currents which were, however, higher than at corresponding voltages on the ascending branch of the plot. A possible explanation for this phenomenon may be that peptides once forced into the conducting state by high voltage do not move back to the non-conducting state until the voltage is considerably lowered. When the orientation of the voltage was changed from −100 mV to +100 mV the membrane current decreased to zero; switching back to

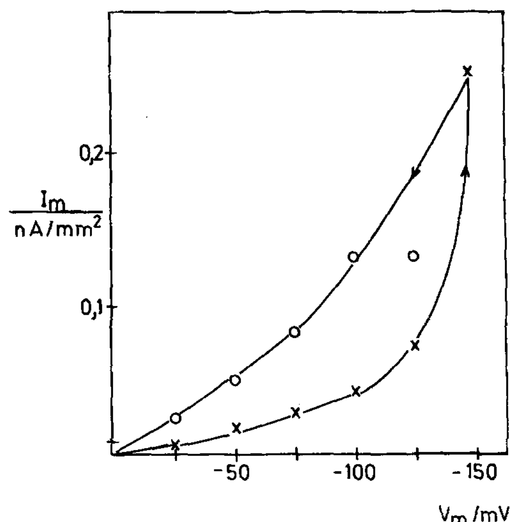

Fig. 3. Current vs. voltage plot of trans-membrane currents across a nisin-doped BLM [DOPC/PS (4:1); area: 1 mm^2). After addition of nisin (0.3 μM) to the cis-side, a transnegative voltage was applied and the resulting currents read after 5 s. The voltage was increased stepwise (*crosses*) and decreased (*open circles*) after reaching −150 mV

−100 mV resulted in an increase of conductivity. However, the increase was considerably slower than the decrease after depolarization. This might indicate that the voltage is necessary for insertion of channels into the membrane and that the peptides are repelled from the membrane due to their hydrophilic nature in the absence of a trans-negative potential.

Single channel recordings

Single channel recordings were performed to better characterize the peptide membrane interaction. For this purpose the bilayer area was reduced to 0.1 mm^2 and the peptide concentration to 100−500 nM. Figure 4 shows sections of charts recorded at −100 mV which are representative for experiments with −50 mV and −150 mV, too. The single channel pattern obtained with nisin differed considerably from the picture known for channel-forming colicins (Pressler et al. 1986). No staircase-like conductance changes of unit size due to insertion of single molecules could be observed.

Instead, very short-lived pulse-like fluctuations stable in the millisecond range were recorded. This picture did not change with the phospholipids and solvents used for membrane formation. Different conductance levels with different life-times could be resolved in Fig. 4. The largest conductance fluctuations had a single channel conductance of approximately 1.2 nS. This value allowed a rough estimate of the maximum diameter of the pores formed by nisin. Assuming that the nisin pore is a hollow cylinder with a length (l) of 6 nm (corresponding to the membrane thickness) and assuming that the pore is filled with an aqueous solution of the same specific conductance (σ) as the bulk phase, the diameter ($d = 2r$) of the pore may be estimated according to

$$\Lambda = \sigma \times \pi r^2 / l\,. \tag{1}$$

Thus, the diameter of the pore with the largest single channel conductance in Fig. 4 (1.2 nS) was about 0.9 nm (using a

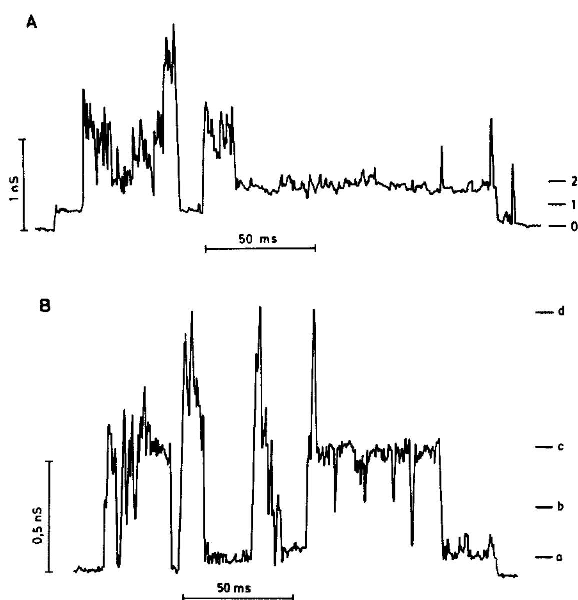

Fig. 4A, B. Nisin evoked conductance fluctuations of a black lipid membrane formed from DOPC/PS (4:1); nisin (100 nM) was added on both sides of the membrane (area: 0.1 mm^2) and the voltage set to −100 mV. On the right different conductance levels are indicated (*1,2* in **A** indicating the more stable low conductance levels and *a*–*d* in **B**)

specific conductivity for a 1 M NaCl solution of σ = 110 mS/cm). This result indicates that the pores formed by nisin are large and permeable for hydrophilic solutes with molecular weights up to 500.

However, the amplitude of single channel fluctuations varied considerably from membrane to membrane and for different membrane potentials, thus making it impossible to assign a defined pore diameter to a certain conductance level. However, it can be stated that the largest pore diameters were up to 1 nm. The high conductance levels (e.g. level *d*, Fig. 4B) were found to be stable only for milliseconds, whereas for lower levels (e.g. levels 1 and 2, Fig. 4A) lifetimes of more than 100 ms could be recorded. It has to be noted that the lifetime for the high levels increased with the increasing membrane potentials. In this respect and in the formation of multi-state pores nisin showed some analogy to the formation of pores by alamethicin (Boheim 1974).

Discussion

The results presented here add further evidence to the model for the primary killing action of nisin proposed previously (Ruhr and Sahl 1985), i.e. membrane disruption in a voltage dependent fashion. The results with intact cells clearly indicate, that the requirement for an energized membrane (Ruhr and Sahl 1985) is indeed a requirement for a transmembrane potential, because K^+ diffusion potentials were sufficient to promote its action. There is a good correlation between these results and those obtained with BLM, thus reducing the possibility of a misinterpretation of the BLM experiments. The BLM experiments proved that the potential must have a trans-negative orientation, i.e. the negative pole must be in the opposite compartment to which the peptide is added; this requirement is fulfilled under physiological conditions of bacterial growth and with valinomycin-induced K^+ diffusion potentials. The BLM experiments also demonstrate that nisin can act on artificial membranes. This question remained open after we failed to influence asolectin vesicles with this peptide (Ruhr and Sahl 1985). However, if nisin needs more than −100 mV to impair an artificial membrane as indicated by the BLM results (Fig. 3), the failure with asolectin is most likely due to insufficient energization of these vesicles. Although, theoretically −120 mV and more can be imposed on such small vesicles by valinomycin-induced K^+ diffusion the magnitude of the potential reached in practice may be lower and most likely not stable enough, to promote nisin action.

The threshold potential necessary for nisin to disrupt physiological membranes is obviously lower than −100 mV, at acidic pH even lower than at neutral pH. The explanation for this phenomenon probably lies in the different composition of artificial and physiological membranes. It must be recalled, that black lipid membranes are composed of a single phospholipid or defined mixtures and contain a considerable amount of solvent. Reisinger et al. (1981) reported on a complex formation of membrane bound undecaprenylpyrophosphate cell wall precursors with nisin. The interaction of nisin with such integral membrane components could facilitate and stabilize nisin pore formation in bacterial membranes, resulting e.g. in lower threshold potentials or longer lifetimes of conducting states.

The single channel recordings obtained for nisin strongly resemble the multi-state pores reported for melittin and alamethicin (Boheim 1974; Hanke et al. 1983). Melittin has been shown to disrupt membranes in a voltage-dependent fashion (Kempf et al. 1982); furthermore it is most likely a tetramer which is involved in pore formation (Tosteson and Tosteson 1984).

The results reported here, strongly suggest a similar mode of action for nisin and probably for many other cationic peptides as outlined elsewhere (Sahl 1985b).

The size of nisin (34 amino acid residues; Gross and Morell 1971) excludes that a single molecule can span a membrane more than once. Therefore, it seems reasonable to assume in analogy to the alamethicin and melittin models that a conducting state represents a pore formed by aggregated peptides. Thus, the formation of oligomers with varying numbers of participating molecules could result in different pore sizes and explain the different conductance levels observed with nisin. However, this working hypothesis needs further experimental proof.

Acknowledgement. The authors would like to thank Angela Schmid and Michaela Josten for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (Sa 292/4-1, 5-1, and Be 865/3-3).

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Received June 23, 1987/Accepted September 3, 1987